THERIOGENOLOGY

ON THE ISOLATION OF EMBRYONIC STEM CELLS: COMPARATIVE BEHAVIOR OF MURINE, PORCINE AND OVINE EMBRYOS

J. A. Piedrahita[1,a], G.B. Anderson[1] and R.H. BonDurant[2]

[1]Department of Animal Science and [2]Department of Reproduction, School of Veterinary Medicine, University of California, Davis, USA

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ABSTRACT

The efficiency of isolation and the characteristics of embryo-derived cell lines from murine, porcine, and ovine embryos cultured on STO feeders or homologous embryonic fibroblasts (HEF) feeders were compared. While murine isolated ICM or intact embryos plated on STO or HEF feeders gave rise to cell lines with embryonic stem cell-like (ES-like) morphology, ovine embryos did not. Cell lines with ES-like morphology were isolated from porcine intact embryos and isolated ICM when plated on STO feeders but not when plated on HEF. Neither murine nor porcine ES-like cell lines expressed cytokeratin 18 or vimentin. Unlike murine ES-like cell lines, porcine ES-like cells did not undergo observable differentiation in vitro or in vivo. Cell lines with epithelial-like morphology were isolated from porcine and ovine embryos. Both porcine and ovine epithelial-like cell lines expressed cytokeratin 18. When induced to differentiate in vitro, porcine and ovine epithelial-like cell lines formed vesicular structures. Electron microscopy revealed that the porcine vesicles were composed of polarized epithelial cells, each with a basally-located nucleus and an apical border containing numerous microvilli with a well organized microfilament core. The results of this study show that conditions which allow isolation of ES cells from murine embryos allow the isolation of porcine embryo-derived cell lines sharing some, but not all, the characteristics of murine ES cells.

Key words: embryonic stem cells, murine, ovine, porcine

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[a]Current address: Department of Pathology, School of Medicine, University of North Carolina, Chapel Hill, NC 27599-7525

INTRODUCTION

Murine embryonic stem (ES) cells, isolated from both whole embryos (1) and isolated ICM (2-4), have similar morphological and biochemical properties to cells from the early murine embryo (5). The ES cells can be maintained in culture in an undifferentiated state and, under appropriate culture conditions, be induced to differentiate in a more or less organized fashion (6). When injected into blastocysts, ES cells have the ability to participate in the formation of all tissues of a chimeric individual, including germ cells (7).

The ability of ES cells to form germ line chimeras has resulted in their use as vectors for the introduction of recombinant DNA into mice. Chimeras produced between genetically modified ES cells and normal embryos have been used to study in vivo gene regulation (8, 9) as well as germ-line transmission of introduced genes (10-13). In addition, ES cells have been used to study targeted modification of genes by homologous recombination (13-17).

Embryonic stem cells have been isolated from murine embryos using either murine primary embryonic fibroblasts (4) or a continuous cell line of murine embryonic fibroblasts (STO) (18) as feeder cells. Recently it has been shown that ES cells can be isolated from hamster embryos using feeders composed of murine primary embryonic fibroblasts (19). Evans et al. (20) have reported the isolation of porcine embryo-derived cell lines with ES-like morphology and a limited ability to differentiate in vitro. Attempts at isolating ovine ES cells by culturing embryos on ovine skin fibroblasts in the presence or absence of Buffalo rat liver (BRL) conditioned media have been unsuccessful (21). The purpose of these experiments was to compare the behavior of murine, ovine, and porcine embryos under conditions known to lead to the production of murine embryonic stem cells. Isolated cell lines were characterized in terms of morphology, in vitro and in vivo differentiative capacity, and expression of the intermediate filaments vimentin and cytokeratin 18. Cytokeratin 18 (22) is also known as cytokeratin Endo B (23), cytoskeletal component X (24), and cytokeratin D (25). Expression of intermediate filaments has been used previously as a marker for early stages of embryonic development (26, 27) and murine embryonal carcinoma (EC) cell differentiation (28, 29). Here we report marked differences in the efficiency of isolation and the characteristics of embryo-derived cell lines from murine, porcine, and ovine embryos cultured on STO feeders or homologous embryonic fibroblast (HEF) feeders.

MATERIALS AND METHODS

Selection of Coat-Color and Isozyme Markers

The murine lines chosen differed in coat color and glucose phosphate isomerase (GPI; E.C. 5.3.1.9) isotype. Embryos obtained from C57/hg (30) and JU (31) females were used to produce ES cell lines. The C57/hg line had a nonagouti, black coat and GPI-1A isotype. The JU line was homozygous for the recessive pink-eye dilute gene. The JU animals were screened for GPI isotype and only those expressing GPI-1B were used.

Blastocyst-stage embryos obtained from JU animals were injected with ES cell lines derived from C57/hg embryos to study in vivo developmental potential.

Purebred Durocs and three-way crossbred pigs were used. Purebred Durocs are characterized by red-brown hair and brown skin pigmentation. The three-way crossbreds (Yorkshire, Hampshire, and Duroc breeds) were characterized by either white or black and white skin and hair pigmentation when sired by a Yorkshire or Hampshire boar. Ovine embryos were obtained from various crossbreds of Dorset, Finn, and Rambouillet.

Murine blood samples were analyzed for GPI by starch gel electrophoresis (32). Porcine blood samples were analyzed for GPI and phosphogluconate dehydrogenase (PGD; E.C. 1.1.1.44). PGD was analyzed by a modification of the method of Fildes and Parr (33). Blood samples were collected in a sodium citrate anticoagulant solution and washed once with saline. Erythrocyte samples were hemolyzed with distilled water containing 0.4% NP40 detergent (Sigma, St. Louis, MO) and frozen at -70°C until use. Samples were analyzed using cellulose acetate electrophoresis. Cellulose acetate strips were presoaked for 20 min in 0.01M phosphate buffer (pH 7.0) and allowed to equilibrate for 5 min at 150 volts. After equilibration, 5-μl samples were applied and the gel was run for 90 min at 150 volts. The strip was then stained for 30 to 60 min (33).

Preparation of Feeder Layers

Homologous embryonic fibroblasts (HEF) were obtained from 30-to 40-d-old porcine embryos, 20- to 40-d-old ovine embryos, and 14- to 16-d-old murine embryos. After removal of the head, liver, and heart, the embryos were minced and incubated 45 min at 37°C in a trypsin/EDTA solution (0.5% trypsin/0.2% EDTA; GIBCO, Grand Island, NY). Large cell clumps were removed and the remaining cells plated on 100-mm tissue culture plates. Cells were allowed to grow to confluence and used to prepare feeder layers up to and including the tenth passage. After the tenth passage cells were discarded and new fibroblast cultures prepared. The STO cells (18) and HEF were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% calf serum (GIBCO), 5% fetal bovine serum (GIBCO), glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (100 μg/ml).

Cells harvested by trypsinization for 5-10 min at 37°C were inactivated with 4500 or 6000 Rads and plated in 35-mm tissue culture dishes (Falcon, Becton Dickinson, Lincoln, NJ) coated with gelatin. Coating was accomplished by adding to the dish a solution of 0.1% gelatin with incubation at room temperature for 20 min. Onto each 35-mm plate 1×10^6 STO cells were added. Feeders were used within a week of preparation.

Embryo Collection and ICM Isolation

All embryo culture and embryo manipulations were carried out in DMEM containing 10% fetal bovine serum (GIBCO and Gemini, Calabasas, CA), 10% calf serum (Sigma), L-glutamine (2 mM), b-mercaptoethanol (0.1 mM), penicillin (100 IU/ml), and streptomycin (100 μg/ml), unless otherwise specified. The fetal bovine serum and calf serum used were selected for

their ability to support growth and differentiation of mouse teratocarcinoma stem cells (PSA). This medium is referred to as ES medium hereafter.

Murine blastocysts were collected on the afternoon of the third day following detection of a mating plug, and the inner cell masses were isolated (34). Blastocysts were incubated for 30 min in ES medium containing 2 x 10^{-5}M calcium ionophore A23187 (Sigma). They were then rinsed and incubated in fresh ES medium. The extent of cell lysis of trophectodermal cells was closely monitored and at the appropriate time (30 to 40 min) embryos were cleaned of lysed cells by use of a mouth-operated micropipette and the isolated ICM transferred to a fresh drop of ES medium.

Porcine blastocysts and hatched blastocysts were collected 7 to 8 d after the first day of estrus (first day of estrus = Day 0). Embryos were obtained by flushing uteri collected at slaughter with Dulbecco's phosphate buffered saline (PBS; 500 ml) containing 5% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 ug/ml). Isolated ICM were obtained by immunosurgery (35). Embryos were incubated for 15 to 25 min in a 1/8 dilution of heat-inactivated rabbit anti-porcine antisera, followed by a 10-to 20-min incubation in guinea pig serum (GIBCO), diluted 1/10. Lysed cells were removed by use of a mouth-operated micropipette. Rabbit anti-porcine antisera were prepared from blood collected from rabbits immunized with three weekly injections of 100 x 10^6 porcine spleen cells.

Ovine blastocysts were collected 6 to 7 d after the first day of estrus (first day of estrus = Day 0). Embryos were collected at surgery by flushing each uterine horn with PBS (200 ml) containing 5% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 ug/ml). ICM were either mechanically isolated (36), or isolated by incubating in calcium ionophore as described above.

Isolation of Embryo-Derived Cell Lines

Whole embryos or isolated ICM were plated on STO or HEF feeder layers in ES medium. Approximately 3 to 5 d after plating for murine embryos, and 7 to 10 d after plating for porcine and ovine embryos, growing ICM were dissociated by incubation in trypsin/EDTA (0.5% trypsin/0.2% EDTA; GIBCO) for 3-5 min at 20-25°C and transferred to a fresh feeder layer with the help of a mouth-operated micropipette (pick passage). At 7 to 10 d later, and at 7-to 10-d intervals thereafter, colonies with embryonic stem cell-like or epithelial-like morphology were selected for further passage. Epithelial-like colonies were composed of flat, cuboidal cells arranged in an organized pattern resembling epithelial sheets. Embryonic stem cell-like colonies were composed of round cells each with a large nucleus and one or two prominent nucleoli. The term embryonic stem-like (ES-like) cell is used to distinguish embryo-derived cells based on morphology; it does not imply that the cell line has pluripotential capabilities. When more than 50 colonies were present on a plate, it was incubated with trypsin/EDTA (0.5% trypsin/0.2% EDTA; GIBCO) for 15 to 20 min at 37°C, an equal volume of fresh ES medium added, and the cells removed from the plate and centrifuged at 250 x g for 5 min (mass

passage). The cell pellet was resuspended in fresh ES medium prior to plating on fresh feeder layers. Colony morphology and presence of colonies in the plates were recorded 5 to 7 d after passage for porcine and ovine colonies and 3 to 5 d for murine colonies. If no colonies were observed 7 d after passage for porcine and ovine cell lines, and 5 d for murine cell lines, the cell lines were considered lost. Colonies were subcultured into increasingly larger culture dishes (35 mm and 100 mm). Isolated cell lines were tested for mycoplasma contamination by DNA hybridization using a DNA probe (Gen-Probe, San Diego, CA) specific for mycoplasma and acholeplasma ribosomal RNA (37).

Survival profiles of murine, porcine, and ovine intact embryos and isolated ICM cultured on STO or HEF were compared by the Friedman two-way analysis of variance by rank (38). Multiple-comparisons to determine individual treatment differences were carried out using the formula $|Rj-Rj'| \geq z \sqrt{(bk(k+1)+6)}$ where Rj and Rj' are the jth and j'th treatment rank totals, $z = \alpha + 6$, b=number of passages, and k=number of treatments (38).

In Vitro Differentiation of Isolated Cell Lines

To induce in vitro differentiation, ES-like or epithelial-like cells were seeded onto tissue culture plates in the absence of a feeder layer. After 7 to 10 d of culture for ovine and porcine cell lines, and 3 to 5 d for murine cell lines, colonies were gently dislodged and placed in 35-mm nonadhesive petri plates (Falcon). Suspension cultures were monitored daily and medium was changed every other day. At the initiation of culture, the first overt signs of differentiation, and when no further differentiation could be visually detected, colony samples were obtained, rinsed in PBS, and fixed in 2.5% glutaraldehyde for 1 h at room temperature and 12 to 18 h at 4°C. After fixation, samples were stored in PBS containing 0.02% sodium azide and stored at 4°C for up to 2 wk. Samples to be analyzed by light microscopy were dehydrated with ethanol, embedded in paraffin, and 8-μm sections prepared with a JKB microtome. Sections were stained with eosin and hematoxylin. Samples to be analyzed by electron microscopy were fixed as above, rinsed in 0.1 M cacodylate buffer, and postfixed in 1% osmium tetroxide for 1 h at 4°C. After a 1-min rinse in water, the specimens were stained with 2% aqueous uranyl acetate for 1 h at 4°C. After dehydration in acetone, samples were embedded in epoxy and thick sections cut and stained with methylene blue-azure II. Ultrathin sections cut with a diamond knife were viewed and photographed with a Philips EM 400 electron microscope.

Immunofluorescent Detection of Intermediate Filaments

Cells were plated on sterile glass coverslips in tissue culture plates. When the cells on the coverslips were 50 to 70% confluent, they were fixed in 2% paraformaldehyde and extracted with cold (-20°C) acetone. Replicate coverslips were stained for vimentin and cytokeratin 18 by indirect immunofluorescence.

Cells were incubated in either mouse anti-vimentin (1/100; ICN Immunobiologicals, Irvine, CA) or mouse anti-cytokeratin 18 (1/10; Sigma) antibodies, diluted in PBS containing 0.5% BSA (PBSA), for 1 h at room

temperature. Coverslips were then rinsed three times in PBSA with changes of rinse solution every 10 min, and incubated for 1 h in a 1/250 dilution of biotinylated goat anti-mouse IgG (Bethesda Research Laboratories, Rockville, MD). After rinsing in PBSA three times for 10 min each, the coverslips were incubated with a 1/100 dilution of Texas red-conjugated streptavidin (Vector Laboratories, Burlingame, CA) and incubated for 45 min at room temperature. Coverslips were rinsed in PBSA for 30 min, mounted in 50% glycerol and observed under a fluorescence microscope (Zeiss, Oberkochen, Germany). For each cell line, background or nonspecific fluorescence was determined by staining coverslips as described above except that no primary antibody was added.

In Vivo Differentiation of ES-Like Cell Lines

The ability of murine cell lines to differentiate in vivo was tested by injection into blastocyst-stage embryos. Day-3 blastocysts (day of mating plug detection = Day 0) were injected (39) with 8 to 12 murine ES-like cells. Injected embryos were transferred surgically to the uteri of Day-2 pseudopregnant recipients. Sex and phenotype of pups were recorded at birth and again at weaning.

The ability of porcine ES-like cell lines to differentiate in vivo was determined by subcutaneous injection of 10×10^6 cells into the flanks of athymic mice (5×10^6 per side). Six to 8 wk after injection animals were inspected for the presence of tumors. Porcine ES-like cell lines were also tested for their ability to differentiate when injected into blastocyst-stage embryos. Porcine blastocysts were collected 6 to 7 d after the first day of estrus and approximately 15 to 20 porcine ES-like cells were injected into the blastocoele (39). Injected embryos were transferred to recipients on Days 5 to 6 of their estrous cycles (1 d behind donor embryos). The phenotype of resulting offspring was recorded at birth.

RESULTS

Isolation of Embryo-Derived Cell Lines

The sequence of events leading to the production of embryo-derived colonies was found to be different for the three species examined. Murine intact embryos and isolated ICM behaved similarly when plated on STO and murine embryonic fibroblasts (MEF; Figure 1). Data for JU and C57/hg murine embryos were combined, since no differences were observed between the two lines in either efficiency of embryo-derived cell line isolation or survival profile (data not shown). When murine intact embryos or isolated ICM were plated on STO feeders or MEF, the embryos generally attached 24 to 48 h after plating. After attachment the ICM continued to proliferate until it reached the egg cylinder stage; the ICM then was disaggregated and transferred to a fresh feeder layer. Colonies with ES-like morphology (Figure 2) were usually seen 2 to 3 d after passage. Pick passage of ES-like colonies continued until approximately 50 colonies were present at which time mass passage was initiated. Epithelial-like colonies, presumably of trophoblastic or endodermal origin, were seen for the first three to four passages after which they generally disappeared.

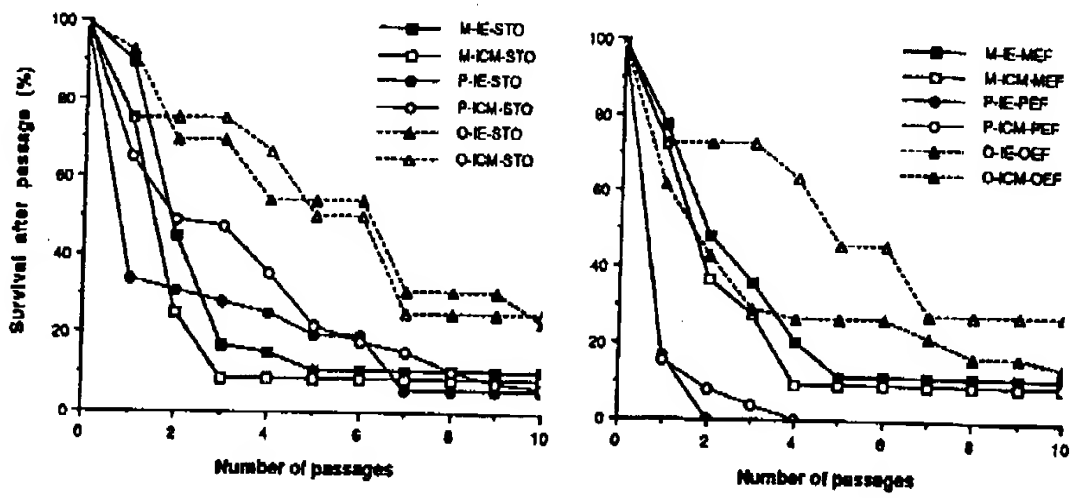

Figure 1. Survival after repeated passage of embryo-derived cell lines from murine (M), porcine (P), and ovine (O) intact embryos (IE) and isolated ICM plated on STO feeders and homologous embryonic fibroblasts (EF).

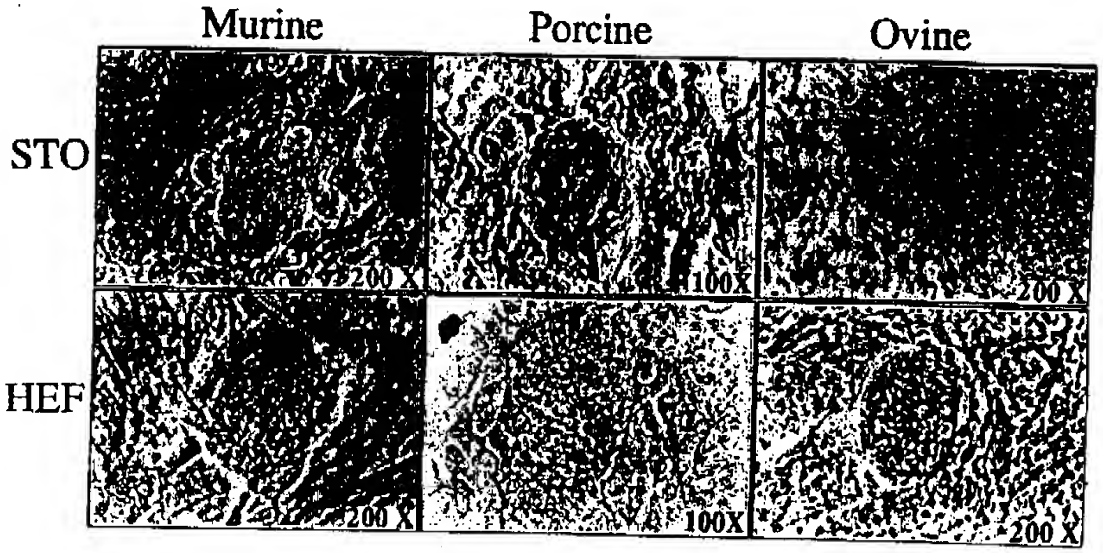

Figure 2. Morphology of ES-like colonies, plated on STO or homologous embryonic fibroblasts (HEF), after repeated passage. Embryo-derived colonies from murine, porcine, and ovine species showed similar morphological characteristics. Each of the colonies showed large cell nuclei with one or two prominent nucleoli except ovine colonies plated on STO, which did not have prominent nucleoli.

We were unable to maintain any murine epithelial-like cell line for greater than 10 passages. Murine ES-like cell lines plated on STO or MEF showed the same efficiency of isolation as well as the same survival profile. Efficiency of isolation of ES-like colonies was 11% from intact embryos cultured on either STO or MEF, and 8 and 9% from isolated ICM cultured on STO and MEF feeders, respectively (Table 1). Although the efficiency of isolation did not differ between embryos cultured on STO versus MEF (Table 1), the cell lines grown on MEF tended to grow slightly faster (5 to 10%, data not shown) and to undergo fewer differentiative changes while plated on MEF than did cells grown on STO. All murine ES-like cell lines had been established by the fifth passage (Figure 1). Furthermore, the survival profile remained relatively unchanged regardless of whether the embryos or isolated ICM were plated on STO or MEF (Figure 1). The established murine cell lines showed a 10-fold population increase in 3 d for an approximate doubling time of 20 h.

Table 1. Comparative behavior of murine, porcine and ovine intact embryos and isolated ICM plated on STO or homologous embryonic fibroblast feeder layers: Number of cell lines surviving greater than 10 passages.

Treatment	Embryos plated	Epithelial-like cell lines	ES-like cell lines	Total isolated cell lines
M-IE-STO[a]	47	0 (0)[b]	5 (11)	5 (11)
M-IE-MEF	98	0 (0)	11 (11)	11 (11)
M-ICM-STO	12	0 (0)	1 (8)	1 (8)
M-ICM-MEF	11	0 (0)	1 (9)	1 (9)
P-IE-STO	36	1 (3)	1 (3)	2 (6)
P-IE-PEF	18	0 (0)	0 (0)	0 (0)
P-ICM-STO	174	6 (3)	5 (3)	11 (6)
P-ICM-PEF	26	0 (0)	0 (0)	0 (0)
O-IE-STO	13	3 (23)	0 (0)	3 (23)
O-IE-OEF	42	6 (14)	0 (0)	6 (14)
O-ICM-STO	12	3 (25)	0 (0)	3 (25)
O-ICM-OEF	11	3 (27)	0 (0)	3 (27)

[a]See figure legend 1 for abbreviations.
[b]Numbers in parentheses are percentages.

Although not statistically different, survival of porcine embryos tended to be longer on STO versus porcine embryonic fibroblasts (PEF). Neither intact embryos nor isolated ICM cultured on PEF survived to the fourth passage. Figure 1 illustrates the rapid decline in surviving cell lines after repeated passage on PEF. When intact embryos or isolated ICM were plated on PEF, only 17 and 15%, respectively, attached to the feeder layer. The remaining embryos either failed to attach or died soon after

attachment. The morphology of the surviving colonies was similar to that of murine and porcine ES-like cell lines (Figure 2). The ICM from colonies that survived plating did not proliferate to a noticeable extent. The ICM attached to the feeder layer and no migration or differentiation was observed (Figure 3).

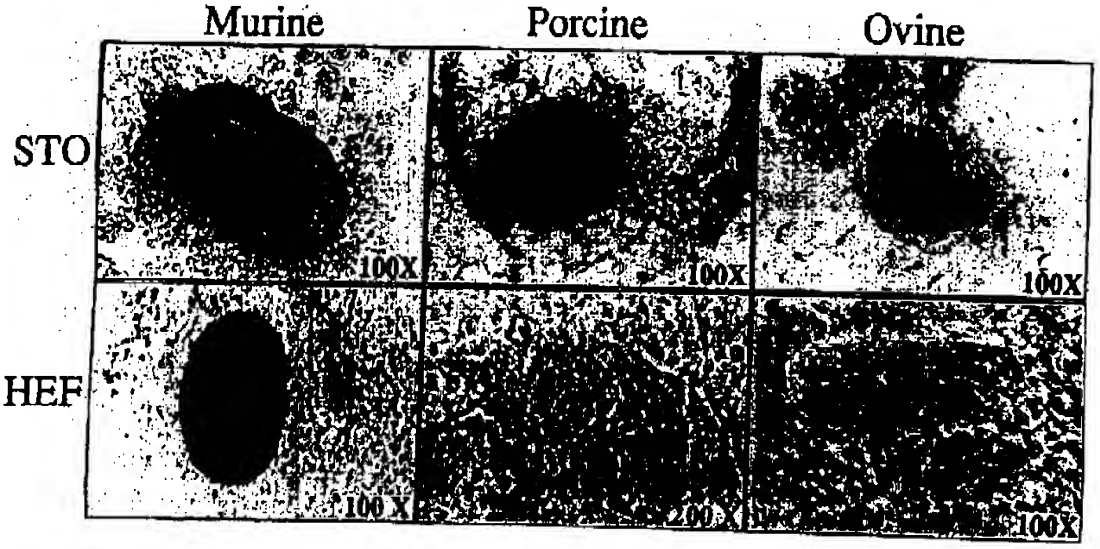

Figure 3. Morphology of ICM 3 to 5 days after attachment to STO or homologous embryonic fibroblast (HEF) feeders. Murine embryos plated on STO and MEF developed into egg cylinders. Porcine ICM proliferated and migrated onto the feeder when plated on STO, but when placed on PEF remained as a well delineated colony with no evidence of proliferation. Ovine ICM both on STO and OEF remained as a cell aggregate with no evidence of spreading onto the feeder. Some ovine embryos plated on OEF differentiated into structures resembling embryonic discs.

In contrast, when intact porcine embryos were plated on STO, they attached to the feeder layer 24 to 48 h after plating. Attachment of the embryo to the feeder layer appeared to be by cells of the ICM, not the trophectoderm; it was difficult to be certain because cell death at the periphery of the ICM did not allow clear visualization of the point of attachment. Three to four days after plating intact embryos, epithelial-like cells, presumably of trophoblast or endoderm origin, or ES-like cells often were seen migrating from the embryo onto the STO feeder layer (Figure 3). The ES-like colonies were allowed to grow for 7 to 10 d before passage. Epithelial-like colonies also were allowed to grow to determine if embryonic stem cells would emerge. If after 14 d no ES-like colonies were observed, the colony was transferred to a fresh feeder layer. The ES-like colonies would sometimes appear for the first time in later passages.

Isolated porcine ICM plated on STO attached to the feeder layer during the first 12 to 24 h in culture. After attachment, cells from the ICM began to spread onto the feeder layer; some cell death was usually

observed at this stage. Thereafter, the ICM either continued to spread or degeneration progressed and the ICM slowly died. Those ICM that continued to spread tended to produce epithelial-like rather than ES-like colonies. After 3 to 8 d of culture, the central portion of the colony (Figure 3) either began a process of degeneration, or a new cell type (ES-like) could be seen migrating from the edge of the ICM. This migration could continue for several days until finally cell degeneration was observed; an attempt was made to pass colonies before this degeneration was seen. In many cases no ES-like cells were observed to originate from the ICM. When this occurred, the colony was passed after approximately 10 d. Commonly, ES-like cells (Figure 2) appeared for the first time at the second, third, or even fourth passage. In addition, we observed two cases in which porcine ICM isolated from 8-d embryos, when cultured on STO feeders, gave rise to colonies of cardiac muscle after 17 to 20 d in culture and five to six passages.

There were no differences in the survival profiles of cell lines isolated from porcine intact embryos or isolated ICM plated on STO ($P>0.05$). There was a gradual decline in the number of surviving cell lines during the first 10 passages. Although epithelial-like cell lines that survived greater than eight passages continued to survive repeated passages (the longest-growing epithelial-like cell line was maintained for 42 passages with no sign of decreased growth rate or obvious morphological changes), the ES-like cell lines remained unstable beyond the tenth passage. The largest number of passages through which an ES-like cell line was maintained was 32 passages over a 6-mo period, at which time the cell line started to degenerate and slowly die, finally disappearing by passage 35. The porcine ES-like cell lines had a much slower growth rate than the murine ES-like cell lines. While the murine cell lines had an approximate population doubling time of 20 h, the apparent doubling time of the porcine ES-like cells approximated 80 h. The actual doubling time was difficult to calculate since the rate of proliferation appeared to be offset by a high rate of cell death. When the growth rate of the porcine epithelial-like cell lines was calculated, it was approximated to be 40 h. Porcine epithelial-like cells did not appear to suffer from a high death rate after passage.

Ovine intact embryos and isolated ICM behaved differently than porcine embryos. Intact ovine embryos attached to the STO or ovine embryonic fibroblast (OEF) feeders 48 to 72 h after plating. Except for a few cases (less than 10%), no growing ICM could be detected in the attached colony. Growth seen in the ovine colonies appeared to be due mostly to growth of the trophoblast, although endoderm migration from the disappearing ICM could not be ruled out. Colonies composed of cells with ES-like morphology (Figure 2) were observed only in embryos with surviving ICM and plated on OEF, and only for a maximum of three passages, after which the ES-like colonies disappeared. Survival profiles of intact ovine embryos tended to be higher than those from murine or porcine intact embryos (Figure 1). Although a high proportion of ovine embryos survived plating and continued passage, the cell lines were slow-growing and unstable. As can be seen from Figure 1, even after 10 passages the cultures did not stabilize but rather continued to disappear. The growth rate of ovine cell lines (40 to 50 h population doubling time) was similar to that of porcine epithelial-like cell lines.

In a few cases murine and porcine ES-like colonies underwent spontaneous morphological changes during culture on STO feeders. Some colonies changed from a uniform group of cells to an irregularly organized mass that formed crater-like structures surrounded by ES-like cells (Figure 4). The changes observed were very similar in both the porcine and murine cell lines. Mycoplasma contamination could not be detected in these spontaneously differentiating cell lines.

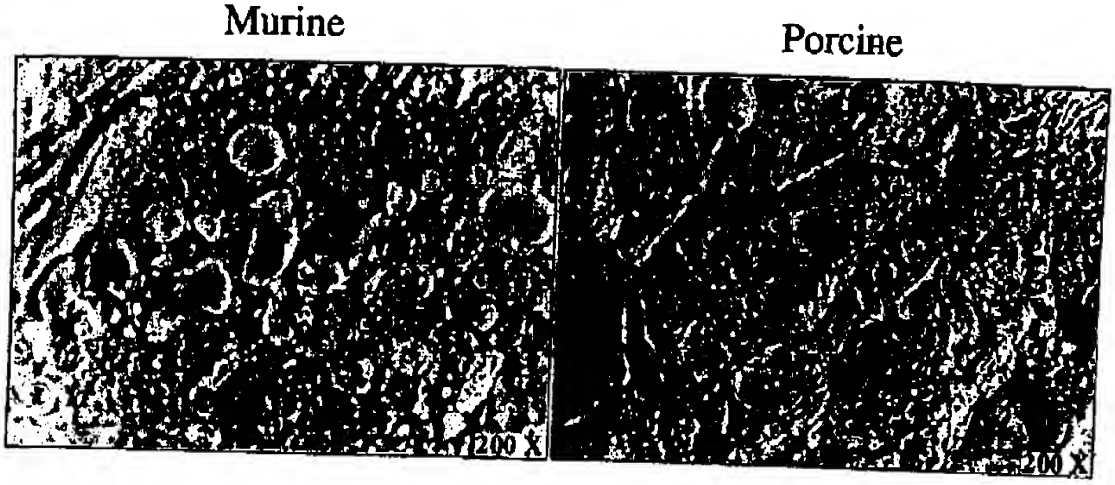

Figure 4. Comparative behavior of spontaneously differentiating ES-like colonies from murine and porcine embryos. Compact, well-delineated colonies plated on STO feeders developed large crater-like spaces surrounded by ES-like cells. The similarity of behavior and morphology of these two cell lines suggests they were of similar type.

In Vitro Differentiation

Murine and porcine ES-like cell lines, and porcine and ovine epithelial-like cell lines, were cultured under conditions known to allow differentiation of murine ES cells. Murine ES cells showed extensive endodermal differentiation, forming embryoid bodies 48 to 72 h after initiation of suspension culture (Figure 5). If kept longer, cystic embryoid bodies were seen beginning 5 to 7 d after initiation of culture. Porcine embryo-derived cell lines with ES-like morphology, in contrast, did not show overt signs of differentiation even after 30 d of suspension culture (data not shown). If kept longer than 30 d the cell aggregates gradually degenerated and died. Unlike porcine ES-like cell lines, however, porcine epithelial-like cell lines showed changes, at least at the light microscopic level, during suspension culture that paralleled those seen in murine ES-like cell lines (Figure 5). Ovine epithelial-like cells formed vesicles 1 to 2 d after initiation of culture. These tended to be disorganized and to exist as groups of vesicles (Figure 6). When the structures formed during in vitro differentiation were analyzed histologically, major species differences were observed.

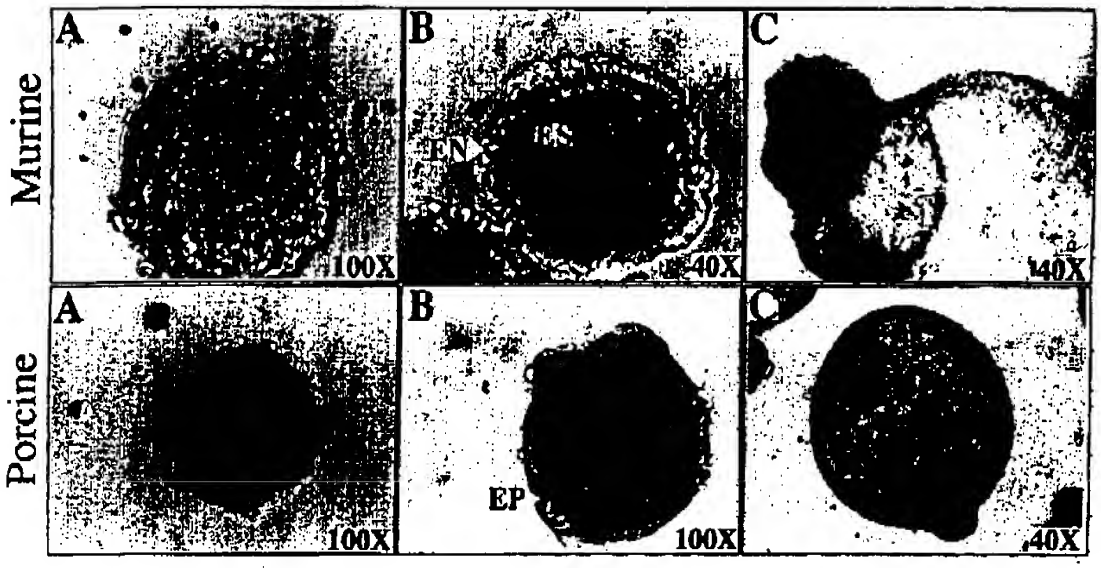

Figure 5. Comparative behavior of ES-like murine and epithelial-like porcine embryo-derived colonies during induced in vitro differentiation. Twenty h after plating (A) no obvious morphological changes were observed. By 48 h each murine colony had a layer of endoderm (EN) surrounding an inner core of ES (B) cells. The porcine colonies also underwent changes and developed an outer epithelial-like (EP) layer. Ten days after initiation of suspension culture (C) both murine and porcine colonies had differentiated into large fluid-containing vesicles.

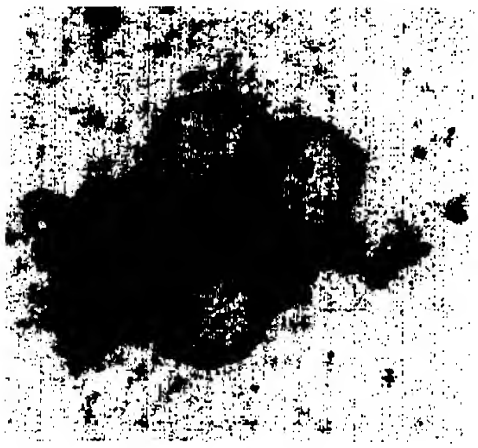

Figure 6. Behavior of an ovine embryonic cell line 3 days after induction of in vitro differentiation. Compared to porcine epithelial-like colonies, ovine colonies were more disorganized; each colony tended to form not one but several vesicular structures. Groups of vesicles were of different sizes and joined by what appeared to be degenerating cells.

While the murine ES-like cell lines showed endoderm layer formation and formation of a cyst surrounded by a double-layered cellular arrangement during differentiation, the porcine and ovine epithelial-like cell lines gave rise to vesiculated structures bounded by epithelial-like cells (Figure 7).

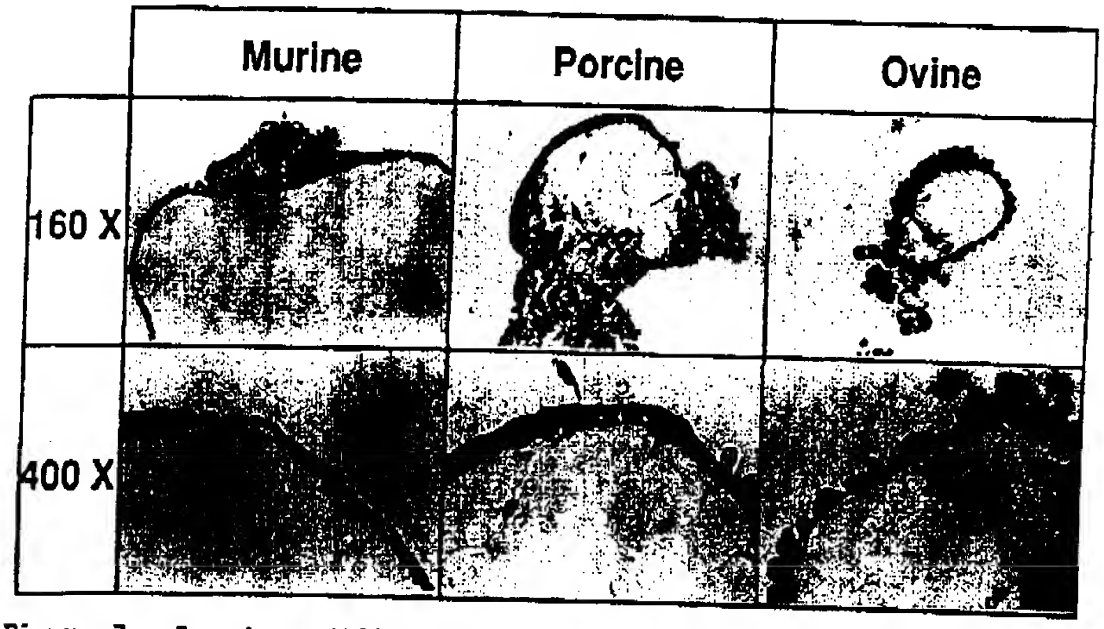

Figure 7. In vitro differentiation of embryo-derived cell lines from different mammalian species: A histological evaluation. Murine ES-like colonies 13 days after initiation of suspension culture each had a large lumen surrounded by a thin double layer of small round cells. Porcine epithelial-like colonies collected 10 days after initiation of suspension culture had differentiated into vesicular structures. The lumen of these vesicles was surrounded by a double layer of columnar epithelium. The ovine epithelial-like cell lines 4 days after initiation of suspension culture also had formed vesicular structures. The wall of the lumen was composed of a single layer of small, round cells differing in size and shape from both porcine and murine colonies.

The walls of murine ES-like embryoid bodies contained numerous nuclei, small flat cells and a two-layered arrangement while the porcine contained pseudostratified columnar epithelial-like cells and fewer nuclei (Figure 7). The ovine-derived cells were more rounded with fewer lateral appositions between cells (Figure 7). When the porcine-derived vesicles were analyzed by electron microscopy, they were found to be composed of polarized epithelial cells each with a basally-located nucleus and an apical border containing numerous microvilli with a well organized microfilament core (Figure 8). The apical-lateral border of the cells showed the presence of junctional complexes. At higher magnification the junctional complexes appeared to be composed of tight junctions and desmosomes. Other characteristics of the cells were their apically

located mitochondria, interdigitating lateral plasma membranes, poorly developed endoplasmic reticulum, and intracellular lipid droplets. Furthermore, in none of the specimens evaluated was the presence of a basement membrane detected.

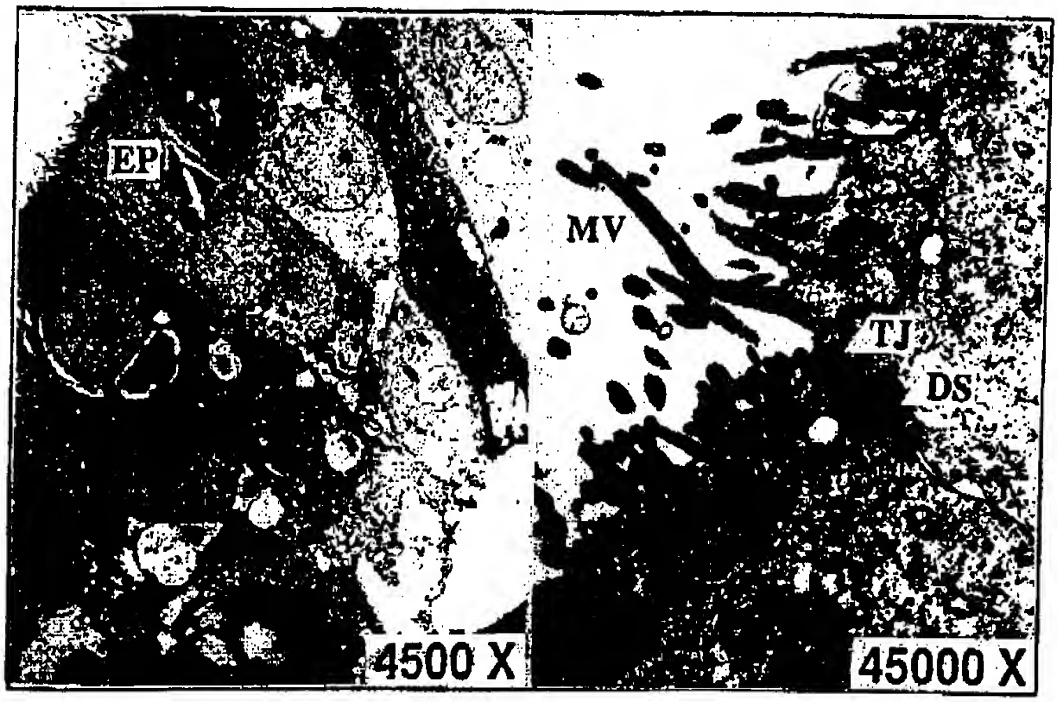

Figure 8. Morphology of a porcine epithelial-like cell line after in vitro differentiation. Electron micrographs of samples collected 7 days after initiation of suspension culture showed a double layer of epithelial cells (EP) resembling stratified columnar epithelium (4,500 X) surrounding the lumen. The epithelial cells had abundant microvilli (MV) in the apical surface and junctional complexes in the apical-lateral area between each cell. When cell-to-cell junctions were observed at higher magnification (45,000 X), the presence of tight junctions (TJ) and desmosomes (DS) was confirmed.

Expression of Intermediate Filaments

Expression of cytokeratin 18 and vimentin in various murine-, ovine-, and porcine-derived cell lines and porcine ICM was examined as a measure of the degree of differentiation in each cell type. Neither vimentin nor cytokeratin 18 was detected in porcine ICM or the cells surrounding the ICM 12 h after plating (data not shown). By 72 h after plating, however, cytokeratin 18 could be detected in the trophectodermal or endodermal cells surrounding the ICM. Cytokeratin 18 but not vimentin was expressed in porcine trophectoderm isolated from Day-9 blastocysts. As expected, neither cytokeratin 18 (Endo B) nor vimentin could be detected in undifferentiated murine ES-like cells (Figures 9 and 10). Although

neither cytokeratin 18 nor vimentin could be detected in porcine cells with ES-like morphology, cytokeratin 18 was detected in porcine epithelial-like colonies (Table 2, Figure 9). Similarly, cytokeratin 18, but not vimentin, was detected in ovine epithelial-like cell lines (Figure 9).

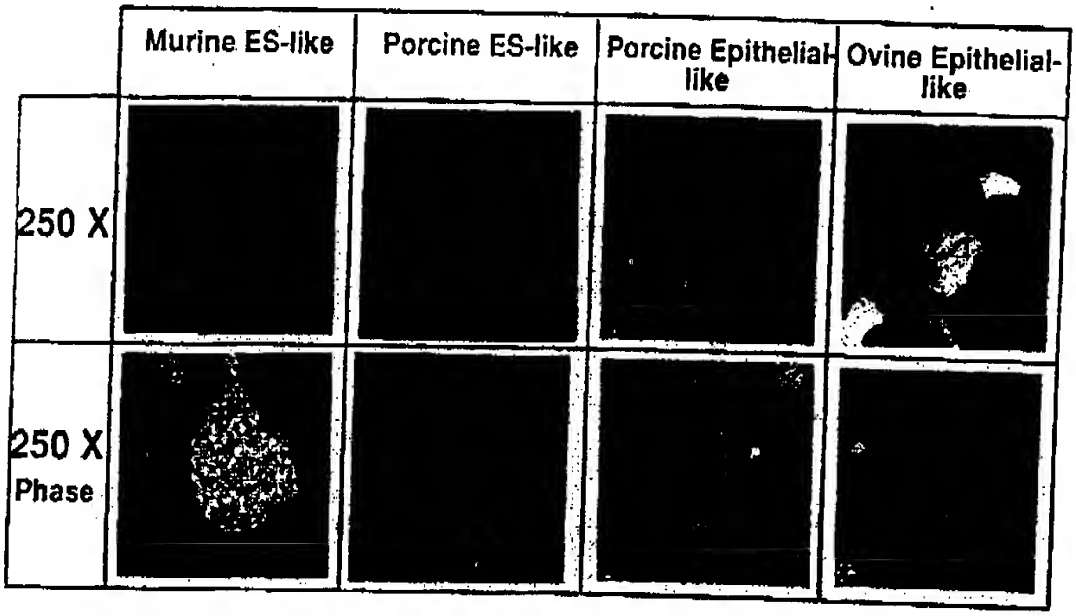

Figure 9. Detection of cytokeratin 18 in embryo-derived cell lines from different mammalian species. Fluorescent and phase contrast photomicrographs showed that murine and porcine ES-like colonies did not express cytokeratin 18. Ovine and porcine epithelial-like cells contained well organized filamentous arrays of cytokeratin 18.

Table 2. Detection of cytokeratin 18 and vimentin in embryo-derived cell lines from different mammalian species and in porcine embryos.

Intermediate filament	Murine ES	Ovine epi-like[a]	Porcine ES-like	Porcine epi-like[a]	Porcine ICM	Porcine trophectoderm	Porcine EF
Cytokeratin 18	-[b]	+	-	+	-	+	-
Vimentin	-	-	-	-	-[c]	-	+

[a] Epithelial-like
[b] Presence (+) or absence (-) of intermediate filaments.
[c] Diffuse, non-filamentous fluorescence was detected.

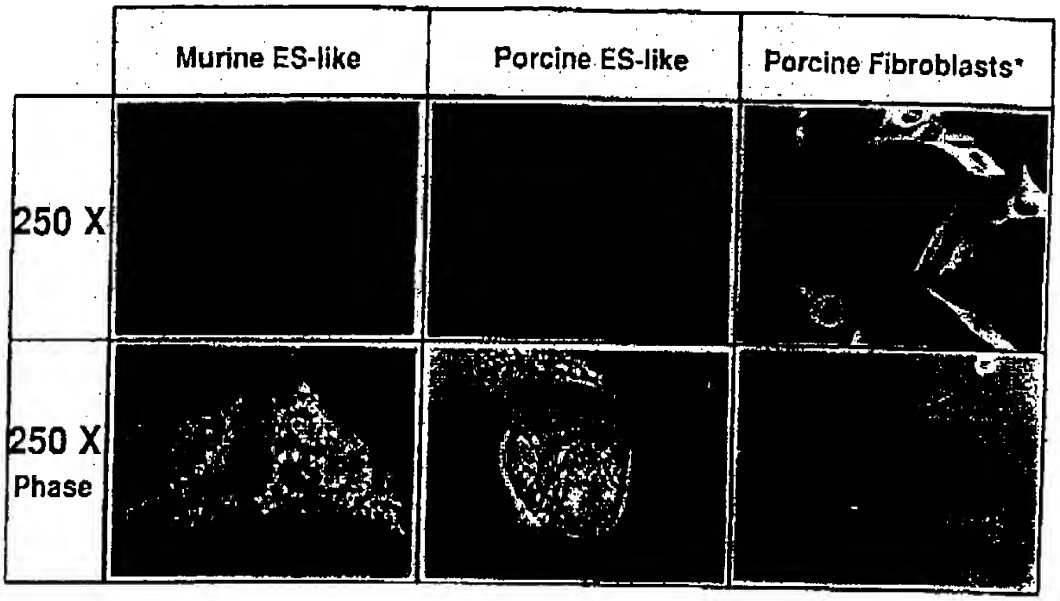

Figure 10. Detection of vimentin in embryo-derived cell lines from different mammalian species. Fluorescent and phase contrast photomicrographs showed that vimentin was not detected in either murine ES-like or porcine ES-like colonies. Ovine and porcine epithelial-like cells were also negative for vimentin (data not shown). Porcine embryonic fibroblasts were positive for vimentin, confirming that the antibodies used detected porcine vimentin.

Porcine and ovine embryonic fibroblasts, examined to determine whether the antibodies used were capable of detecting porcine (Figure 10) and ovine vimentin, were found to be positive for the presence of vimentin. Also, since in some cases the cell lines could have been contaminated with homologous embryonic fibroblasts (HEF) or STO cells originating from the feeder layer, all such cells were examined for the presence of cytokeratin 18. No cytokeratin 18 was detected in either HEF or STO.

In Vivo Differentiation of Murine and Porcine Embryo-Derived Cell Lines

The ability of porcine embryo-derived cell lines to differentiate in vivo was tested by injection of cells into blastocysts for chimera formation and by injection of cells into athymic mice to determine tumor formation ability. Two different cell lines, a cell line with ES-like morphology and an epithelial-like cell line, were injected into two and three athymic mice, respectively. The mice were monitored regularly for the presence of tumors. Eight weeks after injection of cells four of five mice were considered to be devoid of tumor growth. The fifth mouse, injected with an epithelial-like cell line, did contain a tumorous mass. Species origin and tissue type of the tumor were not determined, but the tumor was not located at the site of cell injection, suggesting it had not originated from the injected cells.

Parallel experiments were conducted to determine the ability of porcine embryo-derived cell lines to participate in chimera formation. As summarized (Table 3), 21 piglets were born from sows receiving embryos injected with embryo-derived cell lines with ES-like morphology (P3 and Pm22).

Table 3. Differentiation of porcine embryo-derived cell lines after injection into the blastocoele of normal embryos.

Cell line injected	Embryos injected	Embryos transferred[a]	Pregnant recipient (%)	Piglets born	Chimeras	Embryo survival[b] (%)
P3[c]	47	59	1 (25)	11	0	73
Pm22[c]	58	69	2 (50)	10	0	40
Pk8[d]	9	13	1 (50)	0[e]	0	0

[a] Included injected and non-injected embryos.
[b] Survived to term in pregnant recipients.
[c] ES-like cell line.
[d] Epithelial-like cell line.
[e] Bred recipient gave birth to two piglets, but both were determined not to have originated from transferred embryos (see text for details).

Chimerism was not detected either by coat-color or tissue isozyme markers. Two offspring were obtained from a transfer involving nine embryos injected with embryo-derived cell line with epithelial-like morphology. The animal used as a recipient for these injected embryos was already pregnant from a natural mating. By coat color and isozyme analysis both offspring were determined to originate from the natural mating and not from the transferred embryos.

The ability of murine ES-like cells to differentiate in vivo was tested by injection into blastocysts. From the morphology of the cells (Figure 2), their ability to differentiate in vitro (Figures 5 & 7), and their pattern of intermediate filament expression (Table 2), the injected cell lines were identified as having characteristics similar to those previously described for embryonic stem cells (3). Using coat-color and red blood cell isozyme markers, one of the pups born was determined to be chimeric, confirming the pluripotential character of one of the isolated murine ES cell lines.

DISCUSSION

Murine ES cell lines were isolated from intact embryos and isolated ICM plated on STO or murine embryonic fibroblasts (MEF) feeders. Similar results have been reported by Martin (3), Evans and Kaufman (2), and Wobus

(4). The efficiency of isolation of ES cell lines obtained in this series of experiments compares favorably with that reported by Doetschman et al.(40). Murine ES cell lines that survived greater than five passages became permanently established (Figure 1). Furthermore, the survival profile remained relatively unchanged regardless of whether the embryos or isolated ICM were plated on STO or MEF. Survival profiles were used as basis for comparing the behavior of murine, porcine, and ovine embryos under different experimental conditions. As can be seen from Figure 1, each species had its own unique profile. In the case of ovine embryos the profile within species remained relatively independent of feeder cell type. In the case of porcine embryos there were striking differences in the survival profile depending on the feeder type used as when embryos were plated on STO versus PEF.

In addition to differences in survival profile, differences were observed in type of colonies that could be isolated for each species. Although murine embryo-derived cell lines with morphology and in vitro and in vivo behavior similar to cell lines previously described as ES cell lines (3,41) were isolated, only cell lines with epithelial-like morphology were isolated from ovine embryos. Such cells were most likely of trophoblast or endodermal origin (21), but this has not been unequivocally confirmed. The in vitro behavior of ovine embryo-derived cell lines with epithelial-like morphology differed from that of murine ES cell lines. The structures seen after in vitro differentiation of ovine embryo-derived cell lines resembled structures described as trophoblastic vesicles by Heyman et al. (42, 43). If these structures were trophoblastic vesicles, the ovine cell lines isolated may be useful in coculture experiments since it has been found that coculture of ovine embryos with trophoblastic vesicles results in increased viability of the embryos (43).

The porcine embryo-derived cell lines that were isolated were of two types. Several epithelial-like cell lines with morphology closely resembling the ovine embryo-derived epithelial-like cell lines in addition to ES-like cell lines with a morphology resembling that of murine ES cells were isolated. Attempts to induce porcine embryo-derived cell lines with ES-like morphology to differentiate in vitro did not result in obvious morphological changes. It is not clear why differences are observed between porcine and murine ES-like cell lines in the extent of in vitro differentiation. One explanation is that the trigger for induction of differentiation varies with species. Evans et al. (20) reported induction of in vitro differentiation with porcine embryo-derived cells in media devoid of FBS and β_2-mercaptoethanol. Although we observed no differentiation after induction, morphology of the cells prior to induction of in vitro differentiation (Figure 2), pattern of intermediate filament expression (Table 2), and similarity in behavior of porcine and murine ES-like cells during spontaneous differentiation (Figure 4), strongly suggest that our ES-like cell lines were similar to those isolated by Evans et al.

In culture, murine ES cells and isolated ICM show a similar pattern of differentiation (40, 44, 45). One could speculate that porcine ES cells would differentiate in a manner similar to isolated porcine ICM; unfortunately, there are no previous reports on the behavior of isolated

Snow (52) showed that the division rate is not uniform throughout the primitive ectoderm but that there is a proliferative zone with a significantly faster rate of proliferation. It seems likely that cells giving rise to murine ES cells are primitive ectoderm cells with a high proliferative capacity, perhaps cells from the proliferative zone described by Snow (52). It would seem logical to attempt to determine the equivalent stage at which a high rate of proliferation in porcine and ovine primitive ectoderm occurs. Unfortunately, there are no reports on the pluripotentiality and the growth rate of primitive ectoderm at different stages of embryonic development in porcine and ovine embryos. From our results it appears that expanded blastocysts and early hatched blastocyst stage embryos are not conducive to isolation of ES-like cells with a high rate of proliferation. Whether the difficulties encountered in the isolation of ES cells from porcine and ovine embryos were due to inherent species differences, which make such isolation feasible, or whether the difficulties were due to inappropriate culture conditions or source of embryonic material (e.g., embryos that were too young or too old) remains to be determined.

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